THE REACTIVATION OF INVERTASE

DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN
THE FACULTY OF PURE SCIENCE
COLUMBIA UNIVERSITY

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NEW YORK, N. Y.
1932

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ACKNOWLEDGMENT

For those many enjoyable hours of discussion and of constructive criticism of a chemical philosophy, and for the inspirations received from his direction of this work, the author wishes to express a sincere appreciation to Professor John M. Nelson.

Introduction

Investigators in the field of enzymes have for some time recognized that certain conditions of temperature and acidity seem to destroy or at least inactivate enzymes. In fact it is necessary to take precautions to keep enzymes from being inactivated, so sensitive are they to these conditions.

In surveying the literature it was noticed that occasionally[1,2,3,4,5,6,7] an investigator reported an increase in activity or an apparent regeneration of activity after inactivation. Although it has been mentioned in the literature, reactivation of inactivated enzymes has not been studied very systematically until quite recently. Ohlsson and Swaetichin[7] working on the enzyme amylase in takadiastase, determined the conditions and limitations of reactivation of the taka-amylase after inactivation by acid and by alkali. They gave, however, no explanation as to the nature of the reaction or in what way the enzyme was altered in the processes of inactivation and reactivation.

Reactivation of invertase had been reported by Fodor and Epstein[6] in 1926 with an explanation to account for their results. They obtained reactivation of invertase after an acid inactivation of a yeast macerate but failed to obtain reactivation when a similar procedure was applied to a yeast autolysate. They offered an explanation based on a theory of a transfer of the active part of the enzyme from one carrier to another, by elution and adsorption. They believed that in the yeast macerate that some of the active parts were anchored to a native acid stable carrier while the remaining active groups were attached to breakdown products of the stable carrier and were acid labile. On treating a macerate with acid the active groups on labile carriers were destroyed. On neutralizing the acid, an elution took place of the active groups from the stable carriers onto the free labile carriers. They claimed that the active part of the invertase was more active on the labile carrier than on the stable one so that the elution of the active group from the stable to the labile carrier resulted in an increase in activity or an apparent reactivation of invertase. Now since no increase in activity could be obtained on similarly treating a yeast autolysate they concluded that during the autolysis of the yeast, all the stable carriers were broken down into labile carriers. Therefore after inactivating and neutralizing, there could be no elution and consequently no increase in activity or apparent reactivation.

As will be shown later, in the present work, many different invertase solutions from several different samples of yeast, autolyzed in different ways, could be reactivated after complete inactivation with acid. Therefore the explanation of reactivation by Fodor and Epstein can not be used to account for the phenomenon of reactivation.

Workers in the field of enzymes, with the possible exception of Willstatter[8], believe that enzyme activity is closely tied up with proteins. Many of the properties of enzymes such as, pH-dissociation, temperature coefficient of inactivation, and effects produced by certain heavy metal salts as copper and mercuric chlorides, run parallel to the properties of proteins.

In 1926 Anson and Mirsky[9] published a preliminary notice claiming to have reversed the denaturation of hemoglobin. It was not until 1929[10] that their detailed reports appeared giving their methods. If they denatured hemoglobin in any of several ways and then completely neutralized the solution to pH 6.8, the iso electric point of hemoglobin, the denatured protein was practically completely precipitated. If however the denatured hemoglobin solution was made alkaline to pH 9., where the denatured protein would not flocculate, and was allowed to stand an hour or more, then all of the hemoglobin did not precipitate on returning the solution to the iso electric point. They claimed that in this second case the hemoglobin which remained in solution and did not precipitate out on returning the solution to the iso electric point, had the same properties as the original undenatured hemoglobin.

In surveying the literature it was interesting to note the similarity between the methods used by the various workers to reactivate enzymes and the method reported by Anson and Mirsky[10] for the reversal of denatured hemoglobin. In nearly every instance [1,2,3,4,7] that reactivation of an enzyme was noted the enzyme had been allowed to stand in a solution that was slightly alkaline or less acidic than the pH-activity optimum of the enzyme.

If enzymes are closely allied with or are dependent upon proteins, as thought by many workers in the field, then perhaps the inactivation and recovery of enzymatic activity are dependent on the denaturation and reversal of the protein part of the enzyme. If this were the case then a more precise method of following denaturation and reversal reactions of proteins might be developed from a study of the changes in activity of an enzyme during inactivation and reactivation. Invertase would be particularly suited for such a study for not only minute changes in the extent of invertase activity, but the quality of the activity, as well, can be measured experimentally and quite precisely.*

Since so many factors such as time, temperature, and pH, affect inactivation which in turn affects reactivation, and reactivation itself is conditioned by these variables, it is necessary to hold as many of them constant as is possible during inactivation and reactivation while varying one, if any conclusions are to be drawn from the results.

Experimental Procedures of Present Work

In order to give the reader some idea of the methods used in attacking these problems, a few of the procedures will be described.

The amounts or activities of the invertase solutions used in these experiments were estimated by the rates of hydrolysis of 16 percent sucrose solutions.

In performing an inactivation and reactivation experiment on an invertase solution the following procedure was used. Any deviation from this procedure will be discussed in the details just preceding the experiment. The

* Work along the line already discussed was in progress when Northrop published his papers on crystalline pepsin[11] and on the reversal of denatured inactive pepsin.[12] Work was continued on invertase, however, for there are several points of attack which could still be made with invertase which were not made by Northrop on pepsin.

procedure which was used most often consists of three parts: first, the inactivation part; second, the control experiment; and third the reactivation part. In the first part the inactivation was performed by adding 5.0 cc. of 0.5 M hydrochloric acid to 25 cc. of the invertase solution to be tested. This solution, at pH 1.2 was allowed to stand 5 minutes which time was sufficient to bring about complete inactivation. After the 5 minutes, 5 cc. of approximately 0.5 M sodium hydroxide was added to the inactivated invertase solution, the pH of the solution changing to pH 4.5. As will be shown later, no reactivation occurs at pH 4.5 so that a determination of the activity of this inactivated invertase solution will show the completeness or extent of the inactivation. The activity of this solution was now determined after adding 25 cc. of the inactivated invertase solution to 100 cc. of 20 percent sucrose solution buffered with 0.01 M acetate buffer at pH 4.5, by determining the initial velocity of inversion of the sucrose. In the second part, the activity of the original invertase solution was determined before inactivation and under conditions of dilution similar to the conditions of the first part. This was done by adding 10 cc. of water to the 25 cc. of invertase solution instead of 5 cc. of hydrochloric acid and 5 cc. of sodium hydroxide. The activity was again determined from the initial velocity after adding 25 cc. of this diluted enzyme solution to 100 cc. of 20 percent sucrose solution as in the first part. In the third part of this plan 25 cc. of the original invertase solution was inactivated in the same way as it was done in the first part. Then instead of bringing the solution to pH 4.5, the alkali concentration was adjusted so that the 5 cc. of alkali changed the pH to 6.0–7.0 as was desired for reactivation. This solution at pH 6.0–7.0 was allowed to stand for 30 minutes for reactivation of the invertase to take place after which 25 cc. of this reactivated enzyme was added to 100 cc. of 20 percent sucrose solution buffered with acetate buffer so that the resulting solution was at pH 4.5. From the initial velocity of this hydrolysis the activity of the reactivated invertase was found.

Thus from the results of the first part the activity of the invertase solution after inactivation and before reactivation, was known. The results of the second part gave the activity before inactivation and the third part showed the activity after the reactivation. By comparing the results of these three determinations on equal portions of the same invertase solution, the percent reactivation of the enzyme was found.

Effect of the pH of the Reactivating Medium on the Extent of the Reactivation

In most of the instances in the literature[2,3,4,7] which report reactivation of enzymes, it has usually been reported that complete neutralization of the inactivating acid or alkali did not give as extensive a reactivation as if incompletely neutralized. Tichmorov[2] showed that neutralization of nine-tenths of the inactivating alkali was conducive to reactivation of 10–25 percent of his pepsin, whereas neutralization of all of the inactivating alkali yielded practically no reactivation. Lenard[4], working with the same enyzme, reported that neutralization of seven-eighths of the inactivating alkali gave

the maximum reactivation. The amount of alkali is not so important as is the fact that the workers recognized that the incompleteness of neutralization had a decidedly favorable effect on the reactivation. Gramenitzkii[3] found that heat inactivated taka-amylase recovered most favorably in a 0.0025 percent alkali solution. It remained, however, for Ohlsson and Swaetichin[7] working on taka-amylase to show that it was the pH of the solution in which the enzyme was reactivating that was the important factor in its reactivation. They determined the extent of reactivation at various pHs and obtained a pH-reactivation curve with a maximum at pH 6.4 when the inactivation took place in either acid or alkaline solution. This reactivation maximum at pH 6.4 is on the alkaline side of the pH-activity optimum of pH 5.4 (Ohlsson's value) for taka-amylase. Northrop[12] similarly determined the reactivation of pepsin at different pHs and found the maximum reactivation to take place at pH 5.4. This again is on the alkaline side of the iso electric point and the pH-activity optimum of pepsin which he found to be pH 2.7.

All these procedures are fundamentally similar to the procedure found by Anson and Mirsky[10] to be optimal for the reversal of hemoglobin denaturation. Anson and Mirsky showed that if the denatured hemoglobin solution was made just alkaline enough, pH 9., to prevent flocculation of the denatured protein, which would take place if the acid used to bring about denaturation were exactly neutralized, that approximately 75 percent of the denatured hemoglobin was reversed in an hour. They did not report reversal at other pH values so that it is not known just how reversal of hemoglobin varies with pH. In some more recent work[13] they have been able to obtain reversal of denaturation of hemoglobin by neutralizing only three-fourths of the inactivating acid and allowing to stand for a few hours. Their yield, however, was not as high in this case of allowing the reversal to take place on the acid side of the iso-electric point as when reversal was allowed to take place on the alkaline side.

If invertase is reactivated in a manner similar to that of the other enzymes which have evidenced reactivation, then it may be expected to have a pH-reactivation maximum at some point on the alkaline side of the pH-activity optimum which is pH 4.5 for yeast invertase. Since Anson and Mirsky[13] were able to obtain reversal of denatured hemoglobin on the acid side of the iso-electric point as well as the alkaline side, it was of interest to see if a reactivation of invertase occurred on the acid side of the pH-activity optimum as well as the alkaline. Reactivation of an enzyme by allowing it to stand at a pH slightly more acid than the pH-activity optimum has not been reported in the literature.

In determining the pH to which an inactivated invertase solution must be adjusted for the maximum reactivation to take place, equal portions of an invertase solution were mixed with hydrochloric acid. The invertase in the solution at pH 1.2 was completely inactive after 5 minutes. The portions of the invertase solution which up to this point had been treated in the same way were adjusted to different pHs by adding the same amount, but differing concentrations, of alkali to each solution. These solutions were allowed to reac-

tivate in these media of different pHs for 30 minutes after which their activity was determined. Holding all of the variables such as time and temperature constant and varying the pH at which the enzyme was allowed to reactivate made it possible to attribute the difference in extent of regeneration of activity to the difference in the pH of the reactivating medium. Reactivation was allowed to take place at pHs varying from pH 2.8 to pH 8.0.

The results of these experiments are given in Table A, Part I, with the actual measurements in Table 1, at the back of this thesis. The results are given in graphical form in Curve I of Figure I.

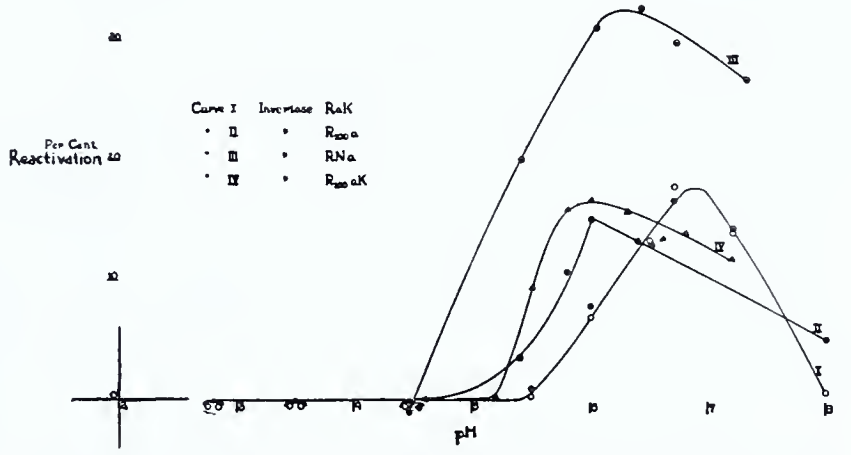

Fig. 1. Effect of the pH of the reactivating medium on the extent of the reactivation of four different invertase preparations.

TABLE A

EFFECT OF THE pH OF THE REACTIVATING MEDIUM ON THE EXTENT OF THE REACTIVATION

Experimental Procedure

25.0 cc. invertase solution+5.0 cc. 0.5 M HCl; inactivation at pH 1.2 for 5 minutes; 5.0 cc. of approximately 0.5 M NaOH was added, adjusting the solution to the pH desired for reactivation. Time of reactivation = 30 minutes.

Activity of reactivated invertase measured by determining the initial velocity of inversion of a 16 percent sucrose solution; 25.0 cc. of reactivated invertase solution+100.0 cc. of 20 percent sucrose solution buffered at pH 4.5.

Part I

Invertase solution RaK

Velocity before inactivation = 2.90° per minute

pH of reactivating solution	Mean values of initial velocities in deg. per min.[1]	Percent Reactivation	Duplicate determinations in deg. per min.	Percent Reactivation
2.8	0.000	—	0.000	—
3.5	0.000	—	0.000	—
4.5	0.000	—	0.000	—
5.5	0.013	0.5	0.034	1.1
6.0	0.204	7.0	0.241	8.3
6.5	0.390	13.5	0.387	13.4
6.7	0.524	18.0	—	—
6.8	—	—	0.490	16.9
7.2	0.411	14.2	0.417	14.4
8.0	0.022	0.9	—	—

Part II
Invertase solution $R_{100}a$
Velocity before inactivation = 1.60° per minute

pH of reactivating solution	Mean values of initial velocities in deg. per minute[1]	Percent Reactivation
4.5	0.000	—
5.4	0.058	3.6
5.8	0.173	10.8
6.0	0.243	15.2
6.4	0.216	13.5
8.0	0.085	5.3

Part III
Invertase solution RNa
Velocity before inactivation = 1.60° per minute

4.5	0.000	0.0
5.4	0.320	20.0
6.1	0.500	31.2
6.4	0.525	32.8
6.7	0.481	30.0
7.3	0.432	27.0

Part IV
Invertase solution $R_{100}aK$
Velocity before inactivation = 2.30° per minute

4.6	0.002	0.1
5.2	0.060	0.3
5.5	0.217	9.5
5.8	0.369	16.0
6.0	0.386	16.8
6.3	0.360	15.7
6.6	0.314	13.6
6.8	0.325	14.1
7.2	0.276	12.0

[1] The actual readings and determinations of these experiments in Table A may be found in Table 1 at the end of the thesis.

As the results given in Part I of Table A and in Curve I of Fig. 1., indicate, yeast invertase reactivates on the alkaline side of the pH-activity optimum. The maximum reactivation of this particular yeast invertase solution occurred at pH 6.7. With the methods used, no reactivation could be detected when the invertase solution was allowed to reactivate on the acid side of the pH-activity optimum.

pH-Reactivation Maxima of Different Invertase Preparations

Throughout their entire investigation Ohlsson and Swaetichin[7] used only the taka-amylase as prepared by Parke Davis Co. They made no mention of the purity of the enzyme solution. Northrop[13] used only crystalline pepsin in his study of inactivation and reactivation of pepsin. Therefore these two investigators gave no indications of a variation in the pH-reactivation maximum for different preparations of their enzymes. Since a number of different

invertase preparations from three different types of autolysates, acid, neutral, and diluted acid (see experimental details) could be had, it seemed desirable to see if the method of preparing the enzyme solution affected the pH at which the maximum invertase was reactivated. If they all showed the maximum reactivation at the same pH then it would appear that the method of purification had no effect on the pH at which the yeast invertase evidenced the most reactivation. If the different invertase preparations showed a maximal reactivation at different pHs then it would indicate that either the accompanying material affects the reactivation of the invertase, or that the invertases differed from each other in some way. If the latter were true, it would be a property which had not been noted before and would be of considerable interest, for as is sometimes the case, a deeper insight into the nature of a substance is gained by a study of the variations in properties exhibited by the substance. Therefore invertase solutions from the different autolysates were examined with the view of determining the pH of the medium in which the maximal reactivation occurred. The other variables such as time, temperature, and inactivation procedure were held constant while the pH of the reactivating invertase solutions was varied.

The results of the experiments are given in the four parts of Table A, with the actual measurements in Table 1, at the end of the thesis. Fig. 1 shows the results in graphical form.

From the results of these experiments it may be seen that all the invertase solutions do not show the same pH-reactivation maxima. The four invertase solutions used had pH-reactivation maxima between pH 6.0–7.0, though not all at the same identical point. Thus either the accompanying material present in the invertase solutions affects the reactivation so as to give different maxima, or the invertases themselves differ in this particular property.

It is interesting to note that between pH 6.–7., where the reactivation maxima were noted is also the region of acidity where other unusual properties of yeast invertase are exhibited. Nelson and Palmer[14] reported that yeast invertase would dialyze more readily through a collodion membrane at pH 6.7 than at the pH-activity optimum of yeast invertase at pH 4.5. Simons[15] noted a difference in the invertase-raffinase ratio when he dialyzed or partially inactivated a yeast invertase solution by heating it at pH 6.6–6.8. All these properties or phenomena seem to indicate that yeast invertase is in a state or condition at pH 6.–7., which is markedly different than at pH 4.5, the optimal pH for invertase activity.

Effect of Time of Reactivation on Extent of Reactivation

Ohlsson and Swaetichin[7] reported that most of the taka-amylase was reactivated in the first hour of standing in a medium at pH 6.4. There was a further reactivation of the enzyme as the time of reactivation was extended but the rate of the reactivation was highest in the first hour, after which the rate dropped off rapidly. Northrop[13] on the other hand found practically no reactivation in the first hour and showed that it required 24 hours or more to recover any appreciable peptic activity.

In view of this difference it seemed important to determine the effect that time has upon the reactivation of invertase. To make this determination an invertase solution was inactivated and the conditions adjusted for reactivation. At intervals of time, aliquot portions of the reactivating invertase solution were tested for their activity.

The results, as shown in Table B and in Fig. 2, indicate that the rate of invertase reactivation is high for the first hour, after which the rate drops off gradually. A similar set of experiments were performed on a different invertase solution and the results, as shown by Part II of Table B, were, in effect, the same. Thus the reactivation of invertase varies with time in a fashion more like the phenomenon reported by Ohlsson and Swaetichin for taka-amylase than that noticed by Northrop for pepsin.

It is interesting to note that the reversal of the denaturation of hemoglobin as reported by Anson and Mirsky[11] takes place rapidly in the first hour after which the rate of reversal is much slower. Here again is a marked similarity between the phenomena of denaturation reversal and enzyme reactivation.

TABLE B

EFFECT OF TIME OF REACTIVATION ON EXTENT OF REACTIVATION

Experimental Procedure

To 100.0 cc. of invertase solution was added 20.0 cc. of 0.5 M hydrochloric acid. After 5 minutes 20.0 cc. of approximately 0.5 M sodium hydroxide was added taking the solution to pH 6.–7. At intervals of time 25.0 cc. of reactivating invertase was added to 100.0 cc. of 20 percent sucrose solution buffered so that the final solution was at pH 4.5. Initial velocities of inversion of the sucrose were determined.

Part I

Invertase solution R_{100}aAD; velocity before inactivation = 1.65° per minute; inactivation at pH 1.2; inactivation time = 5 minutes; reactivation at pH 6.7

Part II

Invertase solution RNa; velocity before inactivation = 2.10° per minute; inactivation at pH 1.2; inactivation time = 5 minutes; reactivation at pH 6.3

Time of Reactivation in minutes	Part I		Part II	
	Initial velocities in deg. per min.[1]	Percent Reactivation	Initial velocities in deg. per min.[1]	Percent Reactivation
0	0.001	0.0	—	—
2	—	—	0.006	0.3
15	0.137	8.3	—	—
17	—	—	0.330	15.7
30	0.223	13.5	0.454	21.6
60	0.302	18.3	0.536	25.5
180	0.358	21.7	—	—
200	—	—	0.606	28.9
1200	0.423	25.6	—	—

[1] The actual readings and determination of these initial velocites may be found in Table 2, at the end of this thesis.

EFFECT OF TIME OF INACTIVATION ON EXTENT OF REACTIVATION

It was noticed throughout this work on invertase as well as the work on other enzymes that reactivation of all of the inactivated enzyme was not obtained under any conditions that have so far been used. Anson and

Mirsky[10,11] were not able to reverse all of the denatured hemoglobin. Was the cause of this destruction merely a matter of time of contact with the inactivating medium? If so then on lengthening the time the enzyme is in contact with the inactivating agent should show a decrease in the amount of enzyme that may be reactivated.

Anson and Mirsky[11] showed that it made little difference whether the denatured hemoglobin was allowed to remain in contact with the denaturing acid for three minutes or for eighteen hours. The extent of reversal was about seventy to seventy-five percent in either case. Thus if one may apply this knowledge, gained from the experiments on hemoglobin, to what appears to be a related problem, one would expect that the destruction of yeast invertase would soon reach a limit beyond which there would be very little destruction even on long periods of inactivation.

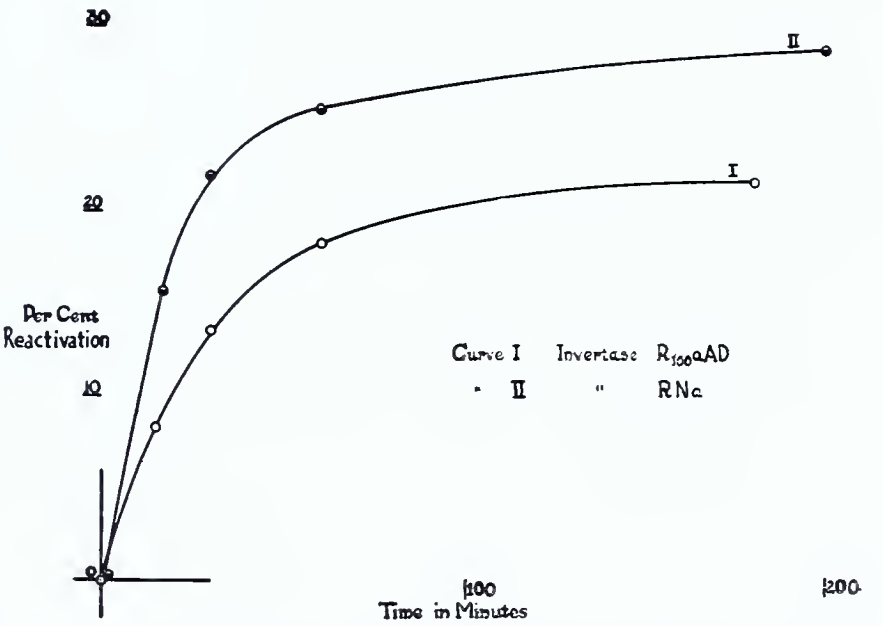

Fig. 2. Effect of the time of reactivation on the extent of reactivation.

To put this idea to test, invertase solutions were inactivated at pH 1.2 for varying lengths of time after which they were allowed to reactivate under identical conditions. By holding the reactivating procedure constant as well as the conditions of temperature and pH of inactivation, the effects produced on the extent of the reactivation of the invertase could be attributed to the variation in the time of inactivation. Two different enzyme solutions were tested in this way and found to be similar in their reactions. The results of these experiments are given in Table C, and in Fig. 3, with the actual measurements being placed in Table 3, at the end of the thesis.

From the results of the experiments, as given in Table C, one may draw a few conclusions. That a destruction or irreversible inactivation takes place very rapidly in the first few minutes of inactivation is shown by the rapid drop in the amount of enzyme that one may reactivate after a few minutes

TABLE C

EFFECT OF TIME OF INACTIVATION ON EXTENT OF REACTIVATION

Experimental Procedure

To 25.0 cc. of invertase solution was added 5.0 cc. of 0.5 M hydrochloric acid. After the desired length of time of inactivation 5.0 cc. of approximately 0.5 M sodium hydroxide was added, taking the solution to pH 6.–7. After 30 minutes of reactivation 25.0 cc. of this invertase solution was added to 100.0 cc. of 20 per cent sucrose solution buffered so that the final solution was at pH 4.5. Initial velocities of inversion of the sucrose were determined.

Part I

Invertase solution RaK; velocity before inactivation = 2.90° per minute; inactivation at pH 1.2; reactivation at pH 6.7 for 30 minutes.

Part II

Invertase solution $R_{100}a$; velocity before inactivation = 2.10° per minute; inactivation at pH 1.2; reactivation at pH 6.4 for 30 minutes.

Time of Inactivation in minutes	Part I		Part II	
	Initial velocities in deg. per min.[1]	Percent Reactivation	Initial velocities deg. per min.[1]	Percent Reactivation
5	0.524	18.2	0.331	15.8
30	0.276	9.4	0.283	13.6
60	—	—	0.251	12.0
180	—	—	0.203	9.7
360	—	—	0.234[2]	11.1
390	0.154	5.2	—	—

[1] The actual determinations of these velocities are in Table 3, at the end of the thesis.
[2] This invertase solution was allowed to reactivate 35 minutes instead of 30 minutes before the velocity was measured.

of inactivation. This destructive reaction apparently arrives at an equilibrium after a short time, for there is very little further destruction as the time of inactivation is lengthened. Thus although it requires only five minutes to

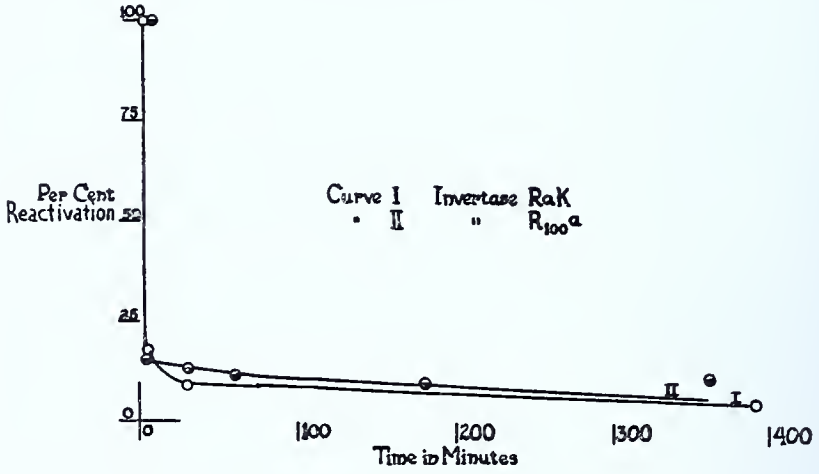

Fig. 3. Effect of the time of inactivation on the extent of reactivation.

inactivate completely the invertase solutions, yet allowing the invertase solution to stand for six hours in the same inactivating medium does not bring about complete destruction of all of the invertase. These findings are somewhat similar or comparable to the those of Anson and Mirsky[11] who showed that reversal of denatured hemoglobin took place to approximately the same

degree whether the hemoglobin was allowed to stand in contact with the denaturing acid three minutes or eighteen hours.

Quality of Action of Reactivated Invertase

Very little work has been carried out with the view to showing how closely the reactivated enzyme resembles the original enzyme. Northrop[13] has shown that the activity per milligram of reactivated crystalline pepsin was identical with the original pepsin he started with. The specific rotation was slightly different but he states that experimental difficulties were such that this difference is still doubtful. Ohlsson and Swaetichin[7] noted a slight shift of the pH-activity optimum of the taka-amylase which had been reactivated. Before inactivation the optimum pH for activity was found to be pH 5.4 while after inactivation and recovery it was pH 5.8.

It is quite conceivable that an alteration in the enzyme unit might be brought about during inactivation, which would not be reversed by reactivation. This alteration might be of such a nature as to affect the quality of the enzymatic action and could be detected only by studying the course of this action. As first pointed out by Nelson and Hitchcock[16] slight differences in invertase action can be detected by applying the empirical equation (I) which describes the course of the hydrolysis of sucrose as represented by the percent

$$(\mathrm{I}) \qquad t=\frac{1}{N}\left(\log\frac{100}{100\text{-}p}\,0.002642\mathrm{p}-0.00000886\mathrm{p}^2-0.0000001034p^3\right)$$

hydrolyzed-time curve. If the values for N are constant to within 0.7 percent throughout the hydrolysis of a 10 percent sucrose solution, the invertase is said to be normal. If the invertase is abnormal it will show either increasing or decreasing values of N as the hydrolysis proceeds. Therefore, if in the process of inactivation, the invertase is altered in some way which affects the quality of its action, and this alteration is not reversed in reactivating the invertase, then it may be detected by running a hydrolysis with this reactivated invertase and studying the course of the hydrolysis by means of the equation (I).

Miss Carmichael[17] has shown that invertase solutions as prepared in these laboratories from Ruppert's yeast give constant values of N, and may be considered as normal. She also showed that these same invertase solutions were retarded in their action to the extent of 78.0 ± 1 percent in the presence of one percent alpha methyl glucoside. In the case of an abnormal invertase or a modified invertase she found a different retardation. This again is a test specific to yeast invertase which may be used to detect slight differences in the action of yeast invertases. With two such means of detecting slight differences in the quality of its action, invertase furnishes a means of comparing the quality of action of a reactivated enzyme with that of a normal or unchanged enzyme.

Three invertase solutions from as many different kinds of autolysates were inactivated completely and then allowed to reactivate for 12 hours. The quality of these reactivated invertase solutions was examined by studying the

courses of their hydrolyzing action, using equation (I), developed by Nelson and Hitchcock.[16]

The retarding effect of one percent alpha methyl glucoside on these same reactivated invertase solutions was studied in an effort to determine if any change had been made in the enzyme on inactivation which could be detected by this means.

The results of these experiments are to be found in Table D, along with the specific directions for performing them.

TABLE D

QUALITY OF ACTION OF REACTIVATED INVERTASE

Experimental Procedures

Inactivation and Reactivation of Invertase. 25.0 cc. of invertase solution+5.0 cc. 0.5 M HCl; inactivation at pH 1.2 for 5 minutes; add 5.0 cc. of approximately 0.5 M NaOH; reactivation at pH 6.7 for 12 hours.

Part I

Values of N. 25.0 cc. of diluted[1] reactivated invertase was added to 100.0 cc. of 12½ percent sucrose solution buffered with 0.01 M Acetate buffer at pH 4.5. 25.0 cc. samples of the hydrolysis mixture were withdrawn at such intervals of time that the entire course of the hydrolysis was represented. These were made alkaline to pH 11., with a drop of saturated alkali to stop the invertase action. The rotation of the samples was determined after mutarotation was at equilibrium.

Part II

Retardation by Alpha methyl glucoside. Initial velocity of the invertase was determined in the presence of one percent alpha methyl glucoside and then in the absence of it, from which results the retardation was determined.

Retardation. 25.0 diluted[1] reactivated invertase+100.0 cc. (12½ sucrose+1.25% alpha methyl glucoside) solution buffered with 0.01 M Acetate buffer at pH 4.5.

Unretarded. 25.0 cc. diluted[1] reactivated invertase+100.0 cc. 12½% sucrose solution buffered at pH 4.5 with 0.01 M Acetate buffer.

a. Invertase solution RaK.
b. Invertase solution RNa.
c. Invertase solution $R_{100}a$.

[1] The reactivated invertase solutions were too active for use in these tests and were diluted so that the activity was within the limits of precise measurement.

Part I
Values of N
a. Invertase Solution RaK

Time in minutes	Rotation	Change in Rotation	Percent inverted	$N \times 10^5$
0	15.43	—	—	—
70	6.70	8.73	43.24	479.0
100	3.78	11.65	57.67	476.4
130	1.43	14.00	69.31	476.3
150	0.21	15.22	75.35	475.1
			mean—	476.7
			a.d.—1.2 or 0.2%	

b. Invertase Solution RNa

0	15.45	—	—	—
19	10.59	4.86	24.06	928.9
37	6.53	8.92	44.16	928.4
55	3.20	12.25	60.64	926.4
80	−0.13	15.58	77.13	930.4
			mean—	928.5
			a.d.—1.1 or 0.1%	

c. Invertase Solution $R_{100}a$

0	15.45	—	—	—
20	10.85	5.60	27.72	1026.0
40	5.06	10.39	51.43	1030.0
53	2.60	12.85	63.61	1028.0
70	0.08	15.37	76.09	1037.0

mean— 1030.0
a.d.—3.0 or 0.3%

Part II

Retardation by One Percent Alpha methyl glucoside

a. *Invertase solution RaK*

1. Unretarded Hydrolysis

Time in minutes	Change in Rotation	Velocity in degrees per minute
20	0.52	0.0260
40	1.04	0.0260
60	1.55	0.0258
80	2.05	0.0256

mean— 0.0258

2. Retarded Hydrolysis

80	0.48	0.00600
160	0.95	0.00594
225	1.31	0.00582
255	1.53	0.00600

mean— 0.00594

77.0% Retardation

b. *Invertase solution RNa*

1. Unretarded Hydrolysis

3	0.50	0.166
6	0.98	0.163
10	1.61	0.161
11	1.80	0.164

mean— 0.164

2. Retarded Hydrolysis

30	1.06	0.0353
39	1.38	0.0356
42	1.48	0.0352
45	1.60	0.0356

mean— 0.0354

78.4% Retardation

c. Invertase solution $R_{100}a$

1. Unretarded Hydrolysis

Time in minutes	Change in Rotation	Velocity in degrees per minute
4	0.61	0.153
6	0.87	0.145
10	1.47	0.147
11	1.65	0.150
		mean— 0.149

2. Retarded Hydrolysis

30	0.93	0.0310
45	1.41	0.0313
52	1.64	0.0315
55	1.76	0.0320
		mean— 0.0315
		78.8% Retardation

As may be seen from Table D, all three the of invertase solutions examined gave constant values for N over the entire course of the hydrolysis of 10 percent sucrose solution. This means that the quality of the invertase action is the same after inactivation and reactivation as it was before this treatment. Thus no change has occurred in the invertase which could be detected in the course of its action.

This might be of importance in other connections, for in the study of enzymes one might be compelled to use strong acid in direct contact with the enzyme. If the activity were desired again it could probably be regenerated, in part at least, but there would always be some doubt in the mind of the worker as to the possibility of the enzyme having been altered by the acid in such a way as to affect the quality of the enzyme as a catalyst. In the case of invertase, however, it must be considered as demonstrated that inactivation and reactivation as outlined do not alter the quality of the invertase as far as we are at present able to ascertain.

The retardation of the reactivated invertase by one percent alpha methyl glucoside was the same as that found by Miss Carmichael[17] and others for normal invertase. This merely confirms the results of the study of the values of N, for as pointed out by Fassnacht[18] when a difference in the enzyme was detected by a change in value of N there was nearly always a change in the retarding effect of one percent alpha methyl glucoside.

Reactivation of Different Invertase Preparations

The experiments included in this division were first started to prove or disprove the conclusion of Fodor and Epstein[6] that invertase from yeast autolysates could not be reactivated after acid inactivation. It was soon discovered that invertase in yeast autolysates could be reactivated after acid inactivation. It was quite possible, however, that reactivation was a property of the exceptional invertase solution and not the general run of preparations.

Thus it was important to determine whether reactivation would take place in the majority or minority of invertase preparations. To test and determine the possibility of invertase reactivation being a property of the abnormal or minority of solutions, a large number of solutions prepared by a variety of different purifying procedures were inactivated and conditions adjusted for reactivation. By using invertase solutions prepared in different ways it was possible to determine whether or not the property of reactivation could be attributed to a particular way of preparing the solution. If reactivation were an attribute of only the occasional invertase solution or of solutions prepared in a particular way then only these enzyme solutions would reactivate while the majority would not.

TABLE E

REACTIVATION OF DIFFERENT INVERTASE PREPARATIONS

Velocity before inactivation. To 25.0 cc. of invertase solution was added 10.0 cc. of water. 25.0 cc. of this solution was added to 100.0 cc. of 20 percent sucrose solution and the initial velocities of inversion were determined at pH 4.5.

Velocity after inactivation or Reactivation at pH 4.5. To 25.0 cc. of invertase solution was added 5.0 cc. of 0.5 M HCl. After 5 minutes at pH 1.2 5.0 cc. of approximately 0.5 M NaOH was added adjusting the solution to pH 4.5. After 30 minutes, 25.0 cc. of this solution was added to 100.0 cc. of 20 percent sucrose solution buffered at pH 4.5 and the initial velocities of inversion were determined.

Velocity after reactivation at pH 6.7. To 25.0 cc. of invertase solution was added 5.0 cc. of 0.5 M HCl. After 5 minutes at pH 1.2, 5.0 cc. of approximately 0.5 M NaOH was added adjusting the solution to pH 6.7 where it was allowed to reactivate for 30 minutes. After this reactivation for thirty minutes 25.0 cc. of this solution was added to 100.0 cc. of 20 percent sucrose solution buffered at pH 4.5 and initial velocities of inversion were determined.

Invertase solution	Time Value[1]	Initial velocities in degrees per minute[2]			Percent Reactivation
		Before Inact.	After Inact.	After React.	
RaK	1.0	2.85	0.000	0.524	18.4
RaKK	0.3	2.40	0.000	0.000	0.0
RNa[3]	14.0	3.10	0.000	1.010	32.2
RNaK[4]	0.5	2.85	0.002	0.650	22.8
RNHDaA[5]		3.87	0.002	0.680	17.7
$R_{100}a$	10.0	1.50	0.001	0.330	22.0
$R_{100}aK$		2.30	0.002	0.330	14.3
$R_{100}aAD$	1.3	1.65	0.000	0.213	12.9
$R_{100}aKAD$	0.5	1.70	0.014	0.023	0.5
$R_{100}aASD$	1.4	0.80	0.002	0.023	2.5
$R_{100}HaDAD$	0.7	2.30	0.003	0.077	3.3

[1] By the term Time Value is meant the time in minutes required for 50 mg. of dry invertase preparation to hydrolyze 25.0 cc. of a 16 percent sucrose solution of pH 4.7 to zero rotation at 25°C.

[2] The actual measurements of the initial velocities may be found in Table 4, at the end of the thesis.

[3] Due to the buffering effect of this invertase preparation RNa, 10.0 cc. of 0.5 M HCl was necessary to make the solution acid to pH 1.2. Accordingly 10.0 cc. of alkali was added to neutralize this acid. The other experiment in which the activity before inactivation was determined, was performed after 20.0 cc. of water had been added instead of the 10.0 cc., so that the dilutions of all three parts were identical.

[4] This invertase solution gave negative results on testing for gums by the Salkowski (Ber. **27**, 497) reduction test, which is sensitive to one part in five thousand. Tests for high molecular weight proteins with trichlor acetic acid and with Esbach reagent, were very weak.

[5] Due to the presence of phosphate salts used in the elution of the invertase from alumina, no time value was determined.

In the experiments, equal portions of each invertase preparation were inactivated and reactivated under identical conditions of time, temperature and pH, in order that differences in the extent of reactivation could be attributed to differences in the invertase solution and not to any variation in the inactivation or reactivation procedures.

As shown by the results of the experiments, given in Table E, the majority of the invertase solutions were definitely reactivated, in part, when treated in the manner as outlined. The results as tabulated lead one to several conclusions. First, that Fodor and Epstein were too inclusive in their conclusion that invertase from autolyzed yeast could not be reactivated after acid inactivation. This is brought out by the fact that most of the solutions from the different autolysates used in the present work demonstrated positively that they could be reactivated. One may also conclude that reactivation is not peculiar to solutions prepared in just one way. The amount of reactivation varied considerably with each solution but no correlation between the extent of reactivation and any property of the solution can as yet be drawn.

Inability to Reactivate at pH 4.5

In surveying the experimental results in Table A, it was noticed that the pH of the solution in which the invertase was reactivating made a profound difference in the extent of the reactivation. At pH 4.5 there was no reactivation of invertase whereas in a more alkaline solution at pH 6.–7, the enzyme evidenced its maximal reactivation.

Since it is possible that the accompanying material in the invertase solution, principally proteinous material, may be denatured by the procedure used in inactivating the enzyme, it might be that the invertase was carried down in the coagulation of this material when the solution was adjusted to pH 4.5. With the enzyme surrounded by proteinous material it is possible to conceive of the enzyme having been removed from its sphere of influence by the coagulation of this extraneous material. This may happen when the acid inactivated enzyme solution is adjusted to pH 4.5 at which point some proteins, such as egg albumin, will coagulate when denatured and then returned to pH 4.5.

This possibility was put to a test by adding some unchanged invertase solution to an acid inactivated invertase solution within thirty seconds after it was adjusted to pH 4.5. It was thought that if the accompanying material coagulated when the inactivated solution was brought to pH 4.5 that it might carry down other enzyme molecules with the inactivated ones. By adding some active invertase solution as soon as possible after the solution had been brought to pH 4.5, it was reasoned that the coagulating protein would carry down some of the added invertase as well as the inactivated invertase, if it carried down any at all. The solution was allowed to stand for thirty minutes to allow for complete coagulation and removal of the enzyme from its field of action. A control experiment was run parallel to the above experiment, water being substituted for the inactivated invertase solution in order that the dilution of the added invertase solution be the same in the control that

it was in the test experiment. Therefore if there was any removal of invertase from the field of action by the inactivated invertase solution, then the activity of that solution should have been less than the activity of the control experiment.

In Table F, Part I, are the results of the hydrolysis to determine the activity or relative invertase content in the solution of inactivated invertase plus the added active invertase. In Part II are the results of the hydrolysis in which the active invertase plus water was used. This was the control ex periment.

TABLE F

INABILITY TO REACTIVATE AT pH 4.5

Part I

25.0 cc. of invertase solution RNaK was made acid to pH 1.2 with 5.0 cc. of 0.5 M HCl for five minutes. After this time 5.0 cc. of approximately 0.5 M NaOH was added to bring the solution to pH 4.5. Within thirty seconds after the addition of the alkali 5.0 cc. of active (same as original) RNaK invertase was added to this inactivated enzyme solution. After standing thirty minutes 25.0 cc. of this mixture was added to 100.0 cc. of 20 percent sucrose solution buffered at pH 4.5. The initial velocity of inversion was determined in the usual manner as described in the experimental details.

Part II

In the control experiment 5.0 cc. of RNaK invertase solution was added to 35.0 cc. of water. 25.0 cc. of this solution was then pipetted into 100.0 cc. of 20 percent sucrose solution, and the initial velocities determined.

Part I

Time in minutes	Change in Rotation	Velocity in degrees per minute
1	0.51	0.510
2	1.03	0.515
3	1.53	0.510
4	2.05	0.512
		mean— 0.512
	Part II	
1	0.52	0.520
2	1.01	0.505
3	1.53	0.510
4	2.00	0.500
		mean— 0.511

The results as given in Table F indicate that thirty seconds after neutralizing the acid inactivated invertase solution there was no lessening of activity of that solution. It is possible that coagulation of the protein, if any, was complete in less than thirty seconds, or that the reaction preventing the invertase from reactivating had reached equilibrium within the thirty seconds after the solution was brought to pH 4.5. Another possibility is that the invertase is dependent upon a proteinous material which is denatured by the process of inactivation of the invertase and coagulates at pH 4.5. If this should prove to be true, then a liberation from this coagulum or a reactivation of the invertase would not be expected under these conditions which are so unfavorable to the reversal of coagulation.

Reactivation after Alkaline Inactivation

Up to this point in the investigation reactivation has been from acid inactivated invertase solutions. Since invertase is inactivated by alkali as well as by acid and both Northrop[13] and Ohlsson and Swaetichin[7] were able to reactivate their enzymes after inactivation with aklali, it was expected that invertase would likewise reactivate after an alkaline inactivation.

In Table G are the results of the experiments on this point. Two different invertase preparations were inactivated at pH 12.1–.2 for five minutes. After that time they were brought to the pH desired for reactivation. When the inactivated invertase solution was brought to pH 4.5 and allowed to stand, no reactivation took place as may be seen in subdivision *a* in Table G. When the invertase solutions were adjusted to pH 6.7 and allowed to stand, reactivation took place. A reactivation of approximately 18 percent of the original amount of invertase occurred in the first thirty minutes of standing. Since two different yeast invertase preparations were used and the results were comparable, it may be presumed that the phenomenon is not characteristic of only an occasional or abnormal yeast invertase solution.

Table G

Reactivation after Alkaline Inactivation

Part I

To 25.0 cc. of invertase solution RNa was added 5.0 cc. of 0.5 M NaOH, the pH of the solution changing to pH 12.2. After 5 minutes 5.0 cc. of approximately 0.5 M HCl was added changing the solution to the pH desired for reactivation. Reactivation was allowed to take place for 30 minutes. The velocity of the invertase solution before inactivation was 3.90° per minute. The activity after reactivation was determined in the usual way by adding 25.0 cc. of the reactivated invertase solution to 100.0 cc. of 20 percent sucrose solution buffered at pH 4.5, and determining the initial velocity of inversion.

a. Reactivation at pH 4.5

Time in minutes	Change in Rotation	Velocity in degrees per minute
2	0.07	0.035
25	0.88	0.035
45	1.69	0.038
65	2.51	0.038
		mean—0.037

b. Reactivation at pH 6.7

1	0.76	0.760
2	1.53	0.765
3	2.30	0.767
4	3.08	0.770
		mean—0.766

Part II

To 25.0 cc. of invertase solution RNHDaA was added 5.0 cc. of 0.25 M NaOH, the pH of the solution changing to pH 12.1. After 5 minutes 5.0 cc. of approximately 0.25 M HCl was added changing the solution to the pH desired for reactivation. Reactivation was allowed to take place for 30 minutes after which the activity was determined. The velocity of this reactivated invertase was determined after adding 25.0 cc. of it to 100.0 cc. of 20 percent sucrose solution buffered at pH 4.5. The activity was determined as initial velocities of inversion of the sucrose. The velocity of the invertase before inactivation was 3.80 degrees per minute.

a. Reactivation at pH 4.5

Time in minutes	Change in Rotation	Velocity in degrees per minute
2	0.15	0.075
4	0.30	0.075
6	0.41	0.068[1]
8	0.58	0.073
		mean—0.075

b. Reactivation at pH 6.7

1	0.31	0.310
2	0.57	0.285
3	0.89	0.297
4	1.22	0.305
		mean—0.299

[1] This value was not used in determining the mean value of the velocities for it deviates from the mean value of the other values more than four times the average deviation of the other values.

Reactivation after Heat Inactivation

It was mentioned in the introduction that an increase in the temperature seems to increase the rate of inactivation of enzymes. The heat, however, may exert an independent influence other than the acceleration of the inactivation reaction. Since it is not known whether heat inactivation and acid inactivation affect the enzyme in the same way it seemed important to ascertain whether reactivation of a heat inactivated invertase solution were possible. It may be that invertase, inactivated by heat, might be changed in some manner that would render the invertase incapable of reactivating.

Two different invertase preparations were inactivated by heating to 90–100°C. They were then subjected to the same reactivating procedure that was used to reactivate the invertase after acid and alkali inactivations. In performing the heat inactivation experiments on these invertase solutions it was necessary to make the solutions slightly acid, pH 3.0. This was necessary to prevent flocculation of the inactivated invertase, which might have taken place if the heating had been carried on at pH 4.5. The inactivation was brought about by plunging the container of the invertase solution, a 250 cc. Erlenmeyer flask, into boiling water and keeping it there for three minutes. The solution was kept in constant motion and the temperature rose to above 90°C. in the first minute. After the three minutes in the boiling water the solution was adjusted to pH 4.5 or pH 6.7 and then brought to 25°C. in a water thermostat, where it was allowed to remain for reactivation to take place.

The results of the experiments are given in Table H.

TABLE H

REACTIVATION AFTER HEAT INACTIVATION

Experimental Procedure

Inactivation. To 25.0 cc. of invertase solution was added 1.0 cc. of 0.5 M HCl in a 250.0 cc. Erlenmeyer flask. This was plunged into boiling water and kept in constant motion.

pH = 3.0; temperature = 90–100°C.; time = 3 minutes.

Reactivation. To the inactivated invertase solution was added 1.5 cc. of approximately 0.5 M NaOH. Cooled in a water bath at 25°C.

pH = *a.* 4.5, *b.* 6.7; temperature = 25.0 °C.

The activity was determined after adding 25.0 cc. of reactivated invertase solution to 100.0 cc. of 20 percent sucrose solution at pH 4.5 and finding the initial velocity.

Part I

Invertase solution RNa; Activity before inactivation = 3.90° per minute

a. Reactivation at pH 4.5 for 30 minutes

Time in minutes	Change in Rotation	Velocity in degrees per min.
5	0.00	—
30	0.00	—
100	0.00	—
b. Reactivation at pH 6.7 for 30 minutes		
5	0.60	0.132
13	1.79	0.138
16	2.20	0.138
22	3.10	0.141

Part II

Invertase solution RNaK Velocity before inactivation = 2.80° per minute

a. Reactivation at pH 4.5 for 150 minutes

Time in minutes	Change in Rotation	Velocity in degrees per min.
5	0.00	—
30	0.00	—
60	0.00	—
b. Reactivation at pH 6.7 for 150 minutes		
10	1.30	0.130
15	2.00	0.133
35	5.64	0.161
40	6.56	0.164
		mean—0.147

From the experimental results, given in Table H, on the reactivation of heat inactivated yeast invertase, one may conclude that reactivation of yeast invertase is possible after such heat inactivation as is described. There was practically no reactivation at pH 4.5, as may be seen by examining subdivision *a* of Table H. In these respects reactivation of heat inactivated invertase resembles the reactivation of both acid and alkali inactivated invertase. Two different invertase preparations were reactivated after heat inactivation which minimizes the possibility of the phenomenon being a peculiarity of an occasional yeast invertase preparation.

Anson and Mirsky[19] point out an interesting item in this connection in a review of their work on denaturation reversal. The rates of inactivation of

some enzymes, the killing of certain bacteria, and the denaturation of proteins all seem to have nearly the same extremely high temperature coefficient. It is the highest temperature coefficient known, according to Northrop[12] and is about 600 for 10°C. Anson and Mirsky[19] believe that in each of the other two cases mentioned above that the inactivation or killing is due to a denaturation of a protein by heat.

Experimental Details

Measurement of Activity

The relative activities of the invertase solutions were computed throughout this work by the method of initial velocities as used by Michaelis and Menten.[20] The curvature of the percent hydrolyzed-time curve is so slight during the first 10 percent of the hydrolysis of sucrose that the tangent to the curve at the origin is practically asymptotic out to 10 percent of completion. Therefore the velocities during the first 10 percent of the hydrolysis are constant for all practical purposes. By taking several samples of the hydrolyzing mixture before the hydrolysis has reached 10 percent of completion, several determinations may be made of the velocity of action, from which an arithmetic mean value may be obtained. This method has the added advantage of determining the velocity of the hydrolysis in the first part of the hydrolysis where there is a relatively small amount of invert sugar to retard the action of the invertase.

The experimental procedure for the determination of initial velocities of inversion of sucrose was as follows: 25.0 cc. of the invertase solution, to be tested, was added to 100.0 cc. of 20 percent sucrose solution buffered with 0.01 M acetate buffer at pH 4.5; 25.0 cc. samples of the hydrolyzing mixture were withdrawn at intervals and the invertase action stopped by the addition of a drop of saturated alkali which adjusted the pH of the sample to pH 11.–12 The alkali also increased the rate of mutarotation of the invert sugar. By examining these solutions in a polariscope the extent of the hydrolysis was determined. Knowing the time that had elapsed before the sample of the hydrolysis was stopped the velocity was calculated. All reactions and measurements were carried out in a water thermostat at 25.0°C.

Materials

The invertase solutions used in this work were prepared in a number of different ways. The invertase solutions are identified by the procedure used in preparing them. The following key gives the symbols used in naming the enzyme solutions and the procedure used for each symbol.

Key

R = Ruppert's brewery yeast autolyzed with toluene and then filtered. Since acids are produced in the autolysis, it is sometimes known as an acid autolysis. The solution becomes acid to about pH 4.5 during the autolysis.

RN = Ruppert's brewery yeast autolyzed with toluene in the presence of Na_2HPO_4 and NH_4OH to keep the mixture at pH 7.–8., during the autolysis.

R_{100} = Ruppert's brewery yeast autolyzed with toluene after being diluted with two volumes of water. The volume of the toluene was about 10 percent of the volume of the yeast.

a = precipitation of the invertase from solution by the addition of an equal volume of ethyl (95 percent) alcohol at 0°C. The precipitate was filtered off and extracted for several hours in an equal volume of distilled water.

K = adsorption of the invertase to Kaolin at pH 3.0 and 0°C. for approximately 15 minutes, filtered and the residue extracted, a process of elution, in ammonia at pH 8.0 for 30 minutes, after which the solution was filtered and the residue discarded.

A = adsorption of the invertase to alumina at pH 4.5 and 25.°C., the solution filtered and the residue extracted, an elution of the invertase from the alumina, in 1 percent $(NH_4)_2HPO_4$ solution at pH 8.0.

H = precipitation of accompanying material, not invertase, by adjusting the solution to pH 3.5, filtering and discarding the residue, changing the filtrate to pH 4.5 and filtering. The filtrate contains the invertase.

D = dialysis of the invertase solution in a collodion bag against running tap water over night or until no sulphate precipitate appeared on the addition of barium ion.

S = The invertase solution was saturated with ammonium sulphate by stirring for several hours with crystalline ammonium sulphate. The precipitate was filtered off through infusorial earth and discarded The invertase remained in the ammonium sulphate solution.

Hydrogen-ion Concentration

pH measurements were made electrometrically. When the solution to be measured was less than pH 7.5 as indicated by preliminary tests, the electrometric determination was made by the use of a quinhydrone electrode and saturated potassium chloride-calomel half cell. If the solution was alkaline or the pH was greater than pH 7.5 then the measurement was made by the use of a hydrogen electrode and a saturated potassium chloride-calomel half cell. The electrodes were standardized frequently against standard acid. A Leeds and Northrup type K potentiometer was used in making the necessary E.M.F. measurements.

Apparatus

A description of the thermostat and polariscope used in this work is to be found in an article by Nelson and Bloomfield.[21] Nonsol bottles were used in the hydrolytic experiments. The pipettes and measuring flasks were calibrated.

TABLE 1. *Effect of pH of reactivating solution on the extent of reactivation.*

Part I

Invertase Solution RaK

Velocity before inactivation = 2.90° per minute; Inactivation at pH 1.2; Time of inactivation = 5 minutes

Time in minutes	Change in rotation
a. Reactivation at pH 2.8	
2	0.00
5	0.00
15	0.00
30	0.00
Duplicate	
15	0.00
30	0.00
b. Reactivation at pH 3.5	
2	0.00
5	0.00
15	0.00
30	0.00
Duplicate	
15	0.00
30	0.00
c. Reactivation at pH 4.5	
2	0.00
5	0.00
15	0.00
30	0.00
Duplicate	
15	0.00
30	0.00
d. Reactivation at pH 5.5	
3	0.03
7	0.09
15	0.22
30	0.48
Duplicate	
15	0.50
30	1.05
e. Reactivation at pH 6.0	
2	0.38*
4	0.81
7	1.44
10	2.07
Duplicate	
6	1.40
10	2.30
20	4.80
30	7.78
f. Reactivation at pH 6.5	
2	0.75
4	1.56
6	2.40
8	3.15
Duplicate	
2	0.71*
4	1.52
6	2.33
8	3.14
g. Reactivation at pH 6.7	
2	0.97
4	1.95
7	3.44
8	3.97
Duplicate (at pH 6.8)	
2	1.04
4	2.10
6	3.21
8	4.13
h. Reactivation at pH 7.2	
2	0.79
4	1.61
6	2.54
8	3.42
Duplicate	
2	0.81
4	1.66
6	2.53
8	3.41
i. Reactivation at pH 8.0	
2	0.05
4	0.09
6	—
8	0.14

* Indicates values not used in calculating mean

Part II
Invertase Solution $R_{100}a$
Velocity before inactivation = 1.60° per minute

Time in minutes	Change in rotation
a. Reactivation at pH 5.4	
5	0.27
10	0.56
15	0.90
22	1.31
b. Reactivation at pH 5.8	
2	0.30*
4	0.69
6	1.04
8	1.39
c. Reactivation at pH 6.0	
2	0.43*
4	0.95
6	1.46
8	1.99
d. Reactivation at pH 6.4	
2	0.40
4	0.84
6	1.36
8	1.81
e. Reactivation at pH 8.0	
3	0.20
6	0.49
9	0.85
12	1.17

Part III
Invertase Solution RNa
Velocity before inactivation = 1.60 degrees per minute

a. Reactivation at pH 5.4	
2	0.64
4	1.30
7	2.23
9	2.85
b. Reactivation at pH 6.1	
2	1.00
4	1.99
6	3.02
8	3.98
c. Reactivation at pH 6.4	
2	1.05
4	2.10
6	3.15
8	4.23
d. Reactivation at pH 6.7	
2	0.93
4	1.92
6	2.87
8	3.88
e. Reactivation at pH 7.3	
2	0.83
4	1.79
6	2.56
8	3.50

Part IV
Invertase Solution $R_{100}aK$
Velocity before inactivation = 2.30 degrees per minute

a. Reactivation at pH 4.6	
5	0.00
31	.05
50	.08
65	.11
b. Reactivation at pH 5.2	
5	0.30
10	0.60
15	0.89
30	1.81
c. Reactivation at pH 5.5	
5	1.08
10	2.02
13	2.93
15	3.38
d. Reactivation at pH 5.8	
2	0.71
4	1.47
6	2.25
8	3.03
e. Reactivation at pH 6.0	
1	0.34
3	1.14
4	1.56
5	1.94
f. Reactivation at pH 6.3	
1	0.36
2	0.73
3	1.07
4	1.43
g. Reactivation at pH 6.6	
1	0.33
3	0.87
4	1.26
5	1.61
h. Reactivation at pH 6.8	
1	0.31
2	0.65
4	1.32
6	2.01
i. Reactivation at pH 7.2	
2	0.51
4	1.09
6	1.70
9	2.65

TABLE 2. *Effect of time of reactivation on the extent of reactivation*

Part I

Invertase Solution $R_{100}aAD$

Velocity before inactivation = 1.65° per minute

Inactivation at pH 1.2 Reactivation at pH 6.7

Time of inactivation—5 minutes

Times in minutes	Change in rotation
a. Velocity after 0 minutes reactivation	
35	0.02
120	0.03
430	0.21
b. Velocity after 15 minutes reactivation	
2	0.27
4	0.54
6	0.82
8	1.13
c. Velocity after 30 minutes reactivation	
2	0.43
4	0.90
6	1.36
8	1.81
d. Velocity after 60 minutes reactivation	
2	0.59
4	1.20
6	1.85
8	2.43
e. Velocity after 180 minutes reactivation	
2	0.69
4	1.42
6	2.16
8	2.97
f. Velocity after 1200 minutes reactivation	
2	0.81
4	1.69
6	2.56
8	3.37

Part II

Invertase Solution RNa

Velocity before inactivation = 2.10° per minute

Inactivation at pH 1.2 Reactivation at pH 6.3

Time of inactivation—5 minutes

a. Velocity after 2 minutes reactivation	
5	0.01
11	0.03
35	0.28
60	0.75
b. Velocity after 17 minutes reactivation	
2	0.63
4	1.33
6	1.98
8	2.68
c. Velocity after 30 minutes reactivation	
2	0.91
4	1.79
6	2.71
8	3.68
d. Velocity after 60 minutes reactivation	
1	0.53
2	1.06
3	1.62
4	2.17
e. Velocity after 200 minutes reactivation	
1	0.59
2	1.21
3	1.85
4	2.44

TABLE 3. *Effect of time of inactivation on the extent of reactivation.*

Part I

Invertase Solution RaK

Velocity before inactivation = 2.90° per minute

Inactivation at pH 1.2 Reactivation at pH 6.7

Time of reactivation = 30 minutes

a. Activity regenerated after 5 minutes of inactivation	
2	1.04
4	2.10
6	3.21
8	4.13
b. Activity regenerated after 30 minutes of inactivation	
2	0.52
4	1.12
6	1.67
10	2.84
c. Activity regenerated after 390 minutes of inactivation	
6	0.91
15	2.26
25	3.86
30	4.66

Part II
Invertase Solution $R_{100}a$
Velocity before inactivation = 2.10° per minute
Inactivation at pH 1.2 Reactivation at pH 6.4
Time of reactivation = 30 minutes

Time in minutes	Change in rotation
a. Activity regenerated after 5 minutes inactivation	
2	0.64*
4	1.32
6	1.99
8	2.65
b. Activity regenerated after 30 minutes inactivation	
6	1.69
10	2.83
14	3.99
17	4.64
c. Activity regenerated after 60 minutes inactivation	
3	0.72*
6	1.50
10	2.50
14	3.54
d. Activity regenerated after 180 minutes inactivation	
2	0.40
4	0.80
6	1.23
8	1.65
e. Activity regenerated in 30 minutes after 360 minutes inactivation	
5	1.10
10	2.32
15	3.59
19	4.63

TABLE 4. *Reactivation of different invertase preparations.*

General procedure for inactivation and regeneration:
Inactivation at pH 1.2
Time of inactivation = 5 minutes
Reactivation at pH 4.5 and 6.7
Time of reactivation = 30 minutes
Activity determined as initial velocity of inversion of 16% sucrose solution at pH 4.5
a. = Reactivation at pH 4.5
b. = Reactivation at pH 6.7

Part I
Invertase Solution RaK
Velocity before inactivation = 2.85° per minute

a.	2	0.00	b.	2	1.04
	4	0.00		4	2.10
	6	0.00		6	3.21
	8	0.00		8	4.13

Part II
Invertase Solution RaKK
Velocity before inactivation = 2.40° per minute

a.	2	0.00	b.	2	0.00
	4	0.00		4	0.00
	6	0.00		6	0.00
	8	0.00		8	0.00

Part III
Invertase Solution RNa
Velocity before inactivation = 3.10° per minute

Note: Invertase Solution RNa had such a strong buffering effect that 10.0 cc. of 0.5 M HCl was necessary to make the solution acidic to pH 1.2. Accordingly, 10.0 cc. of alkali was added to neutralize the acid. The velocity of the invertase solution before inactivation was determined under conditions of dilution similar to those used in the above reactivation experiment.

a.	2	0.00	b.	1	1.01
	4	0.00		2	2.03
	6	0.00		3	3.03
	8	0.00		4	4.03

Part IV
Invertase Solution RNaK
Velocity before inactivation = 2.85° per minute

a.	5	0.00	b.	2	1.29
	26	0.06		4	2.60
	60	0.11		5	3.27
	100	0.27		6	3.90

Part V
Invertase Solution RNHDaA
Velocity before inactivation = 3.87° per minute

a.	10	0.00	b.	5	3.37
	30	0.04		8	5.44
	60	0.11		9	6.16
	210	0.41		9.5	6.47

Part VI
Invertase Solution $R_{100}a$
Velocity before inactivation = 1.50° per minute

a.	10	0.00	b.	2	0.64*
	18	0.00		4	1.32
	42	0.03		6	1.99
	50	0.05		8	2.65

Part VII
Invertase Solution $R_{100}aK$
Velocity before inactivation = 2.30° per minute

a.	5	0.00	b.	1	0.31
	31	0.05		2	0.65
	50	0.08		4	1.32
	65	0.11		6	2.01

Part VIII
Invertase Solution $R_{100}aAD$
Velocity before inactivation = 1.65 degrees per minute

a.	35	0.02	b.	5	0.99
	120	0.03		10	2.09
	430	0.21		18	4.00
				20	4.47

Part IX
Invertase Solution $R_{100}aKAD$
Velocity before inactivation = 1.70 degrees per minute

a.	5	0.04*	b.	2	0.02*
	15	0.21		4	0.10
	40	0.54		7	0.16
	60	0.84		9	0.20

Part X
Invertase Solution $R_{100}aASD$
Velocity before inactivation = 0.80 degrees per minute

a.	30	0.06	b.	10	0.20
	210	0.42		35	0.73
	425	0.93		90	2.15
				125	3.10

Part XI
Invertase Solution $R_{100}HaDAD$
Velocity before inactivation = 2.30 degrees per minute

a.	15	0.06	b.	2	0.13
	30	0.09		5	0.36
	60	0.12		10	0.79
	120	0.33		20	1.81

Summary

1. The majority of yeast invertase preparations from three different types of autolysates were partially reactivated after complete inactivation in acid at pH 1.2.

2. A reactivation of invertase after complete inactivation by acid at pH 1.2 took place when the medium had a pH 5.8–7.2, whereas the author was unable to obtain any reactivation at the pH-activity optimum of invertase, pH 4.5.

3. The pH at which the maximum reactivation occurred was not always the same for all invertase preparations. The pH-reactivation maxima for the invertase preparations used in this work lay between pH 5.8–7.0.

4. The amount of reactivatable invertase decreased as time of inactivation increased up to approximately 30 minutes. After 30 minutes the amount of reactivatable invertase remained relatively constant as time of inactivation was extended.

5. Reactivated yeast invertase was found to be normal invertase, as shown by the constancy of N in the equation of Nelson and Hitchcock which describes the course of the hydrolysis. The retardation of the reactivated invertase by one percent alpha methyl glucoside was $78 \pm 1.$ percent, which is that known for normal invertase.

6. Invertase was reactivated after complete inactivation with alkali at pH 12.1. Reactivation occurred at pH 6.7, whereas no reactivation of invertase occurred at pH 4.5.

7. Yeast invertase, inactivated completely by heating for three minutes at 90–100°C., at pH 3.0, was reactivated in part by allowing the inactivated invertase solution to stand at 25°C., in a medium at pH 6.7. No reactivation took place when the medium had a pH of 4.5.

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VITA

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